FORM-PTO-1390 Rev 10-96) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 001560-381

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5): Unassigned 09/530260

NTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP99/04653	27 August 1999	28 August 1998

TITLE OF INVENTION
PROCESS FOR PRODUCING ARACHIDONIC ACID-CONTAINING LIPID AND DIHOMO-γ-LINOLENIC ACID-CONTAINING LIPID

APPLICANT(S) FOR DO/EO/US
Kengo AKIMOTO, Kenichi HIGASHIYAMA and Sakayu SHIMIZU

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1 [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2 [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1).

4. [ ] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.

8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. [X] An unexecuted oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.

14. [ ] A substitute specification.

15. [ ] A change of power of attorney and/or address letter.

16. [X] Other items or information:

1. Certificate of Deposit Form Pursuant to the Budapest Treaty for SAM 0219 (FERM BP-1239); and
2. Certificate of Deposit Form Pursuant to the Bodapest Treaty for SAM 2153 (FERM BP-6794).

526 Rec'd PCT/PTO 28 APR 2000

U.S. APPLICATION NO (If known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
Unassigned 09/530260	PCT/JP99/04653	001560-381

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Search Report has been prepared by the EPO or JPO $840.00 (970)		
International preliminary examination fee paid to USPTO (37 CFR 1.482) $670.00 (956)		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) $690.00 (958)		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $970.00 (960)		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $96.00 (962)		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840.00	
Surcharge of **$130.00 (154)** for furnishing the oath or declaration later than 20 ☐ 30 ☐ months from the earliest claimed priority date (37 CFR 1.492(e)).	$ -0-	

Claims	Number Filed	Number Extra	Rate		
Total Claims	22 -20 =	2	X$18.00 (966)	$ 36.00	
Independent Claims	7 -3 =	4	X$78.00 (964)	$ 312.00	
Multiple dependent claim(s) (if applicable)			+$260.00 (968)	$ -0-	
TOTAL OF ABOVE CALCULATIONS =				$ 1,190.00	
Reduction for 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$	-
SUBTOTAL =				$ 1,190.00	
Processing fee of **$130.00 (156)** for furnishing the English translation later than 20 ☐ 30 ☐ months from the earliest claimed priority date (37 CFR 1.492(f)). +				$ -0-	
TOTAL NATIONAL FEE =				$ 1,190.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00 (581)** per property +				$	
TOTAL FEES ENCLOSED =				$ 1,190.00	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $ 1,190.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 02-4800 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4800. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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24,970
REGISTRATION NUMBER

Filed: April 28, 2000

(09/99)

Patent
Attorney's Docket No. 001560-381

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Kengo AKIMOTO et al.	
Application No.: Unassigned	Group Art Unit: Unassigned
Filed: April 27, 2000	Examiner: Unassigned
For: PROCESS FOR PRODUCING ARACHIDONIC ACID-CONTAINING LIPID AND DIHOMO-γ-LINOLENIC ACID-CONTAINING LIPID	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination on the merits, please amend the above-identified application as follows:

IN THE CLAIMS:

In claim 7, line 2, please delete "or 6".

In claim 10, line 2, please delete "or 9".

In claim 17, line 5, please delete "to 16".

In claim 18, line 6, please delete "to 16".

In claim 19, line 2, please delete "or 18".

In claim 20, line 2, please delete "or 18".

Please add new claims 21 and 22 as follows:

--21. An arachidonic acid-containing microbial lipid containing 72% by weight or more of arachidonic acid in fat per total fatty acids in said fat.

22. The arachidonic acid-containing microbial lipid according to claim 21 wherein the percentage of eicosapentaenoic acid to the totalk fatty acids in said lipid is 0.5% by weight or less.--

REMARKS

Entry of the foregoing and favorable consideration of the subject application, in light of the following remarks, are respectfully requested.

By the foregoing amendment, claims 7, 10, 17, 18, 19 and 20 have been amended to delete their multiple dependencies. New claims 21 and 22 have also been added. Support for these new claims can be found throughout the originally filed application. No new matter has been added by the foregoing amendment.

In the event that there are any questions relating to this Preliminary Amendment, or the application in general, it would be appreciated if the Examiner would telephone the

undersigned attorney concerning such questions so that prosecution of this application may be expedited.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: _____
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Date: April 28, 2000

DESCRIPTION

PROCESS FOR PRODUCING ARACHIDONIC ACID-CONTAINING LIPIDS AND DIHOMO-γ-LINOLENIC ACID-CONTAINING LIPIDS

Field of the Invention

The present invention relates to a process for producing lipids containing arachidonic acid or a process for producing lipids containing dihomo-γ-linolenic acid by fermentation using a mutant in which ω3 unsaturation activity is decreased or deleted.

Background Art

There are reports that arachidonic acid, as is docosahexaenoic acid, contained in breast milk and plays a role in the infant's development ("Advances in Polyunsaturated Fatty Acid Research," Elsevier Science Publishers, 1993, pp. 261-264) and reports on the importance of arachidonic acid in the development of fetus's height and brain (Proc. Natl. Acad. Sci. U.S.A., 90, 1073-1077 (1993), Lancet, 344, 1319-1322 (1994)), and accordingly there are moves to supplement infant formula with arachidonic acid and docosahexaenoic acid that represent major differences in the fatty acid composition between breast milk and infant formula.

The FAO/WHO has issued a recommendation that the intake of arachidonic acid and docosahexaenoic acid be 60 mg/kg/day and 40 mg/kg/day, respectively, for permature infants and the intake of arachidonic acid and docosahexaenoic acid be 40 mg/kg/day and 20 mg/kg/day, respectively, for mature infants.

As methods of obtaining these fatty acids in large amount, there are conventionally known production methods that utilize microorganisms. For example, a method utilizing a microorganism belonging to the genus Mortierella has been proposed that permits the production

of arachidonic acid in a shorter culturing time, with a higher yield, and using simpler processes using an inexpensive commonly used medium (Japanese Examined Patent Publication (Kokoku) No. 7-34752).

However, the percentage of arachidonic acid in the total fatty acids is not satisfactory with these conventional production methods. Thus, when lipids containing arachidonic acid are to be added to foods, the highest possible content of arachidonic acid is preferred since it can minimize the amount of undesirable substances added and therefore it is also desirable in terms of quality and cost. It is also desirable when arachidonic acid ethyl esters are to be isolated and purified, since the highest possible content of arachidonic acid providing highly purified products with simple procedures and at low cost.

As methods of enhancing the percentage of arachidonic acid in lipids relative to the total fatty acids, there are many methods known in the art. For example, by culturing Mortierella alpina at 28°C in a normally airation and agitation culture followed by culturing for further 6 days under the condition of complete glucose depletion, the percentage of arachidonic acid has successfully been raised to 67.4% (Appl. Microbial. Biotechnol. 31, 11-6 (1989)). This method, however, takes advantage of the fact that microorganisms in a starved state effect β-oxidation of fatty acids of triglyceride with a low degree of saturation to convert them to energy.

Therefore, there are actually no changes in the total amount of arachidonic acid, and due to the reduction of fatty acids with a low degree of saturation only the relative percentage of arachidonic acid is enhanced. Thus, it does not mean that the amount of produced triglycerides containing a high percentage of arachidonic acid is increased, but on the contrary it appears that the percentage of triglycerides is also

decreased as a result of β-oxidation.

It is known that at a temperature lower than the optimum growth temperature, microorganisms capable of producing arachidonic acid generally try to adapt to low-temperature environments by enhancing the degree of unsaturation of unsaturated fatty acids so as to maintain fluidity and the functions of the cell membrane, and thereby the activity of Δ6 desaturase and Δ5 desaturase become enhanced so that fatty acids having a high degree of unsaturation such as arachidonic acid are produced in greater amounts. Culturing at a low temperature is therefore desirable to enhance the content of arachidonic acid.

In a method utilizing the above property, Mortierella alpina was cultured in an airation and agitation culture at 20°C for 16 days in which the percentage of arachidonic acid was successfully enhanced to 71.2% ("Industrial Applications of Single Cell Oils," American Oil Chemists' Society Champaign, 1992, pp. 52-60). It is known, however, that since this method requires a long time for culturing, it is not only unsuitable for industrial production but at the low temperature a part of arachidonic acid that was produced is converted to eicosapentaenoic acid by a ω3 desaturase (Biochem. Biophys. Res. Commun., 150, 335-341 (1988)) that acts at low temperatures thereby reducing the percentage of arachidonic acid and increasing the percentage of eicosapentaenoic acid in the total fatty acids.

For example, when filamentous fungus of the genus Mortierella is cultured at 12°C for 7 weeks, ω3 desaturase is activated and the percentage of EPA to the total fatty acids reaches 2 to 20% (J. Am. Oil Chem. Soc., 65, 1455-1459 (1988)) and accordingly the percentage of arachidonic acid decreases. In contrast, by using strains wherein ω3 desaturase is decreased or

is lacking, the percentage of arachidonic acid to the total fatty acids in the lipid can be enhanced to 50% or more, and when mutation is repeated it can be enhanced to 70% or more and the percentage of EPA can be kept at 0.5% or less.

Little eicosapentaenoic acid is contained in breast milk, and recent studies have even shown that it is detrimental to the development of babies of premature infants ("Advances in Polyunsaturated Fatty Acid Research," Elsevier Science Publishers, 1993, pp. 261-264). Thus, there is a strong need for the development of a method that can produce lipids containing a high percentage of arachidonic acid and containing little or no eicosapentaenoic acid using an inexpensive commonly used medium, a simple process, and on a large scale.

On the other hand, dihomo-γ-linolenic acid is converted to arachidonic acid by a Δ5 desaturase irrespective of the culture temperature. As a method of producing dihomo-γ-linolenic acid on a large scale by a fermentation at low cost, there is a known method of culturing by adding a substance that inhibits the activity of Δ5 desaturase such as sesamin, episesamin, sesaminol, episesaminol, and curcumin to the medium, or a method of culturing using a mutant strain of a microorganism capable of producing arachidonic acid in which mutation has been induced so that Δ5 desaturase activity is decreased or deleted (Japanese Unexamined Patent Publication (Kokai) No. 1-243992, Japanese Unexamined Patent Publication (Kokai) No. 3-72892, Japanese Unexamined Patent Publication (Kokai) No. 3-49688, and Japanese Unexamined Patent Publication (Kokai) No. 5-91887).

In this case also, however, culturing at a temperature lower than the optimum growth temperature such as 12°C in an attempt to enhance the dihomo-γ-

linolenic acid content would result in the activation of the above-mentioned ω3 desaturase so that concern arises that a part of dihomo-γ-linolenic acid may be converted to 8, 11, 14, 17-eicosatetraenoic acid and thereby the percentage of dihomo-γ-linolenic acid may decrease and that of 8, 11, 14, 17-eicosatetraenoic acid may increase. Thus, there is a strong need for the development of a method that can produce lipids containing a high percentage of dihomo-γ-linolenic acid using an inexpensive commonly used medium, a simple process, and on a large scale.

Disclosure of the Invention

Thus, the present invention is intended to provide a process for producing arachidonic acid-containing lipids containing a high percentage of arachidonic acid and containing little or no eicosapentaenoic acid using an inexpensive conventionally-used medium, a simple process, and on a large scale, and a process for producing lipids containing a high percentage of dihomo-γ-linolenic acid and containing little or no eicosapentaenoic acid or 8, 11, 14, 17-eicosatetraenoic acid using an inexpensive conventionally-used medium, a simple process, and on a large scale.

After extensive research in order to attain the above objectives, the inventors of the present invention have found a microorganism in which ω3 desaturase activity is decreased or deleted and which is obtained by the mutagenesis of a microorganism capable of producing arachidonic acid, and thereby have completed the present invention.

Thus, the present invention is intended to provide a process for producing lipids containing arachidonic acid comprising the steps of culturing a microorganism wherein ω3 desaturase activity has been decreased or is lacking

at a temperature lower than the optimum growth temperature from the start of culturing or after culturing at the optimum growth temperature, said microorganism being obtained by the mutagenesis of a microorganism capable of producing arachidonic acid and belonging to the genus Mortierella, the genus Conidiobolus, the genus Pythium, the genus Phytophthora, the genus Penicillium, the genus Cladosporium, the genus Mucor, the genus Fusarium, the genus Aspergillus, the genus Rhodotorula, the genus Entomophthora, the genus Echinosporangium or the genus Saprolegnia; and then

recovering lipids containing arachidonic acid from the culture.

Furthermore, the present invention is intended to provide a process for producing lipids containing arachidonic acid comprising the steps of culturing a microorganism wherein ω3 desaturase activity has been decreased or is lacking at a temperature lower than 20°C from the start of culturing or after culturing at 20 to 40°C, said, microorganism being obtained by the mutagenesis of a microorganism belonging to the subgenus Mortierella; and then

recovering lipids containing arachidonic acid from the culture.

The present invention is also intended to provide a process for producing lipids containing dihomo-γ-linolenic acid comprising the steps of

culturing a microorganism wherein ω3 desaturase activity has been decreased or is lacking at a temperature lower than the optimum growth temperature from the start of culturing or after culturing at the optimum growth temperature, said microorganism being obtained by the mutagenesis of a microorganism that is capable of producing arachidonic acid in which microorganism Δ5 desaturase activity has been decreased or is lacking, and that belongs to the genus Mortierella,

the genus Conidiobolus, the genus Pythium, the genus Phytophthora, the genus Penicillium, the genus Cladosporium, the genus Mucor, the genus Fusarium, the genus Aspergillus, the genus Rhodotorula, the genus Entomophthora, the genus Echinosporangium or the genus Saprolegnia; and then

recovering lipids containing dihomo-γ-linolenic acid from the culture.

Furthermore, the present invention is intended to provide a process for producing lipids containing dihomo-γ-linolenic acid comprising the steps of

culturing a microorganism wherein ω3 desaturase activity has been decreased or is lacking at a temperature lower than 20°C from the start of culturing or after culturing at 20 to 40°C, said microorganism being obtained by the mutagenesis of a microorganism in which Δ5 desaturase activity has decreased or is lacking, which had been obtained by the mutagenesis of a microorganism belonging to subgenus Mortierella; and then

recovering lipids containing dihomo-γ-linolenic acid from the culture.

Embodiment for Carrying out the Invention

In accordance with the present invention, as microorganisms that are subjected to mutagenesis, there can be mentioned microorganisms belonging to the genus Mortierella, the genus Conidiobolus, the genus Pythium, the genus Phytophthora, the genus Penicillium, the genus Cladosporium, the genus Mucor, the genus Fusarium, the genus Aspergillus, the genus Rhodotorula, the genus Entomophthora, the genus Echinosporangium and the genus Saprolegnia, and more preferably microorganisms belonging to the genus Mortierella, the genus Conidiobolus, the genus Pythium, the genus Entomophthora, the genus Echinosporangium and the genus Saprolegnia that have Δ6

desaturase and Δ5 desaturase and that can produce fatty acids up to arachidonic acid in the fatty acid biosynthetic pathway.

More specifically, there can be mentioned Pythium insidiosm ATCC28251 as a microorganism belonging to the genus Pythium; Echinosporangium transversalis ATCC16960 (NRRL3116) and ATCC18036 (NRRL5525) as a microorganism belonging to the genus Echinosporangium; Saprolegnia ferax CBS534.67, Saprolegnia lapponica CBS284.38, Saprolegnia litoralis CBS535.67, Saprolegnia moniligera CBS558.67, and Saprolegnia turfosa CBS313.82 and the like as a microorganism belonging to the genus Saprolegnia.

Specifically, in accordance with the present invention, microorganisms belonging to the genus Mortierella subgenus Mortierella that have a high ability of producing arachidonic acid are preferred. As microorganism belonging to the genus Mortierella subgenus Mortierella of the present invention, there can be mentioned Mortierella elongata, Mortierella exigua, Mortierella hygrophila, Mortierella alpina, Mortierella parvispora, Mortierella beljakovae, Mortierella globalpina, Mortierella epigama, Mortierella kuhlmanii, Mortierella acrotona, Mortierella zychae, Mortierella rishikesha, Mortierella minutissima, Mortierella bainieri, Mortierella schmuckeri, and the like, and specifically there can be mentioned strains Mortierella elongata IFO8570, Mortierella exigua IFO8571, Mortierella hygrophila IFO5941, Mortierella alpina IFO8568, ATCC16266, ATCC32221, ATCC42430, CBS219.35, CBS224.37, CBS250.53, CBS343.66, CBS527.72, CBS529.72, CBS608.70, and CBS754.68, and the like.

These strains are all available without limitations from the Institute for Fermentation Osaka (IFO), Japan, and American Type Culture Collection (ATCC) in the U.S.A., and Centrralbureau voor Schimmelcultures (CBS). It is also possible to use Mortierella elongata SAM0219 (FERM P-8703) (FERM BP-1239) that is a microbial strain

our research group has isolated from the soil.

In accordance with the present invention, microorganisms that are subjected to mutagenesis are not limited to those wild type strains described above but preferably include mutants or recombinants of the above microorganisms (wild type strains) belonging to the genus Mortierella subgenus Mortierella, in other words those that have been deliberately designed to have a higher content of arachidonic acid or dihomo-γ-linolenic acid in lipids, or a higher content of total lipid than those produced by the original wild type strains, when they are cultured in the presence of the same substrate.

Further included are those microorganisms that were designed to produce unsaturated fatty acids at an amount equal to that produced by the wild type strains by efficiently using substrates having excellent cost effectiveness. For example, as a mutant strain in which Δ12 desaturase activity is lacking and Δ6 desaturase activity has been enhanced, there can be mentioned Mortierella alpina SAM2086 (FERM BP-6032), and as a mutant strain lacking Δ5 desaturase activity that was artificially induced to enhance the productivity of dihomo-γ-linolenic acid, there can be mentioned Mortierella alpina SAM1860 (FERM BP-3589).

In accordance with the present invention, by subjecting mutant strains of the present invention in which ω3 desaturase has been decreased to mutagenesis, it is also possible to obtain mutant strains in which ω3 desaturase activity has been further decreased or is lacking.

ω3 desaturase activity as used herein refers to an action of inserting a double bond in between the third and the fourth carbons from the methyl group of a fatty acid, and microorganisms in which ω3 desaturase activity has been decreased or is lacking can be easily evaluated

for the decrease or lack of ω3 desaturase activity thereof.

Specifically, for the production of lipids containing arachidonic acid, it can be evaluated by the percentage of eicosapentaenoic acid in the total fatty acids in the microbial cells after a mutant that was obtained by the mutagenesis of the parent strain is cultured at a temperature lower than the optimum growth temperature, for example a temperature lower than 20°C. Thus, when the percentages of eicosapentaenoic acid in the parent strain and the mutant strain under culturing at a low temperature are compared and the percentage of eicosapentaenoic acid of the parent strain is set at 1, then if the percentage of the mutant strain is lower than 1, the activity is judged to be decreased, while if it is 0, the activity is judged to be lacking.

For the production of lipids containing dihomo-γ-linolenic acid also, it can be evaluated by the percentage of 8, 11, 14, 17-eicosatetraenoic acid in the total fatty acids in the microbial cells after a mutant that was obtained by the mutagenesis of the parent strain (for example, a parent strain in which Δ5 desaturase activity has been decreased or lacked) was cultured at a temperature lower than the optimum growth temperature, for example a temperature lower than 20°C. Thus, when the percentages of 8, 11, 14, 17-eicosatetraenoic acid in the parent strain and the mutant strain under culturing at a low temperature are compared and the percentage of 8, 11, 14, 17-eicosatetraenoic acid of the parent strain is set at 1, then if the percentage of the mutant strain is lower than 1, the activity is judged to be decreased, while if it is 0, the activity is judged to be lacking.

As microorganisms for use in the process of producing dihomo-γ-linolenic acid of the present invention, mutant strains in which ω3 desaturase

activity has been decreased or is lacking, that are obtained by the mutagenesis of microorganisms capable of producing arachidonic acid can be used, and more preferably mutant strains in which further Δ5 desaturase activity has been decreased or is lacking, are used. In order to obtain mutant strains in which ω3 desaturase activity has been decreased or is lacking and Δ5 desaturase activity also has been decreased or is lacking, a mutant strain obtained according to the present invention in which ω3 desaturase activity has been decreased or lacked is further subjected to mutagenesis and thereby mutant strains in which Δ5 desaturase activity has also been decreased or lacked are selected. Alternatively, they are also obtained by subjecting strains in which Δ5 desaturase activity has been already decreased or is lacking to mutagenesis so as to decrease or delete ω3 desaturase activity.

Mutation treatment according to the present invention can be conducted by conventional mutagenesis: for example effecting irradiation treatment (X ray, gamma ray, neutron beams, heavy ions), ultra violet irradiation, high temperature treatment and the like to induce mutation; and by suspending microorganisms in a suitable buffer, to which a mutagen is added followed by incubating for a given time, which is diluted appropriately and inoculated on an agar medium to obtain colonies of mutant strains.

As mutagens, alkylating agents such as nitrogen mustard, methyl methane sulfonate (MMS), and N-methyl-N-nitro-N-nitrosoguanidine (NTG), base analogs such as 5-bromouracil, antibiotics such as mitomycin C, base synthetic inhibitors such as 6-mercaptopurine, dyes such as proflavine (other derivatives), certain carcinogens such as 4-nitroquinoline-N-oxide, and other compounds such as manganese chloride and formaldehyde, and the like

may be mentioned. Microorganisms used may be live cells (mycelia etc.) or spores.

As mutant strains of the present invention, for example, Mortierella alpina SAM2153 (FERM P-15767) (FERM BP-6794) wherein ω3 desaturase activity has been extremely decreased that was induced by the present inventors from Mortierella alpina IFO8568 capable of producing arachidonic acid can be used, but it is not limiting said strain, and all other mutant strains that exhibit the ratio of an activity of smaller than 1 relative to eicosapentaenoic acid of the parent strain cultured under a low-temperature condition set as 1.

In order to culture the microbial strains for use in the present invention, spores, mycelia, or a preculture that has been previously cultured are inoculated to liquid media or solid media and are cultured. In the case of liquid media, carbon sources include, but not limited to, any of glucose, fructose, xylose, saccharose, maltose, soluble starch, molasses, glycerol, mannitol and the like that are commonly used. As nitrogen sources, in addition to natural nitrogen sources such as peptone, yeast extract, malt extract, meat extract, casamino acid, corn steep liquor, soy flour, defatted soybean meal and cottonseed meal, defined organic nitrogen sources such as urea and inorganic nitrogen sources such as sodium nitrate, ammonium nitrate, and ammonium sulfate can be used. For large scale industrial production of fatty acids or lipids containing them, liquid media are preferably used.

When desired, inorganic salts such as phosphates, magnesium sulfate, iron sulfate, and copper sulfate, and vitamins can also be used as trace nutrients. The concentrations of these medium components are not limited as long as they do not adversely affect microbial growth. Generally, from a practical viewpoint, carbon sources are in the range of 0.1 to 40% by weight and preferably 1 to 25% by weight, nitrogen sources are in the range of 0.01

to 10% by weight and preferably 0.1 to 10% by weight, and more preferably the initial amount added of carbon sources is 1 to 5% by weight, and that of nitrogen sources is 0.1 to 6% by weight and, during culturing, carbon sources and nitrogen sources, and more preferably only carbon sources, are fed.

The mutant strains of the present invention may be cultured at a temperature lower than the optimum growth temperature from the start of the culturing, or after culturing at the optimum growth temperature they may be cultured at a temperature lower than the optimum growth temperature. Though the optimum growth temperature as used herein may vary depending on the microorganism used, it is preferably 20 to 40°C and preferably 20 to 30°C, and a temperature lower than the optimum growth temperature is a temperature lower than 25°C, preferably a temperature lower than 20°C, and more preferably a temperature lower than 20°C and higher than 5°C. By means of the temperature control described above, the accumulation of lipids in the cells can be enhanced.

When culturing is effected at a temperature lower than the optimum growth temperature, culturing is conducted for 2 to 20 days, preferably 2 to 14 days. When culturing is conducted at the optimum growth temperature prior to culturing at a temperature lower than the optimum growth temperature, culturing is conducted for 1 to 6 days and preferably 1 to 4 days at the optimum growth temperature, and 2 to 14 days and preferably 2 to 10 days at a temperature lower than the optimum growth temperature.

pH of the medium is 4 to 10 and preferably 6 to 9, and airation and agitation culture, shaking culture, or stationary culture is conducted.

When cultured in a solid culture, bran, chaff, rice bran or the like to which 50 to 100% by weight of water relative to the weight of the solid has been added is used, and cultured at the above temperature for 3 to 14

days. In this case, nitrogen sources, inorganic salts, and trace nutrients may be added as desired.

Furthermore, in accordance with the present invention, a substrate for biosynthesis for arachidonic acid or dihomo-γ-linolenic acid may be added to a medium to facilitate the accumulation of arachidonic acid or dihomo-γ-linolenic acid. Examples of substrates for biosynthesis include hydrocarbons such as tetradecane, hexadecane, and octadecane, fatty acids such as tetradecanoic acid, hexadecanoic acid, and octadecanoic acid or salts (e.g. sodium salts and potassium salts) or esters thereof, or lipids containing fatty acids as components (e.g. olive oil, coconut oil, and palm oil).

Arachidonic acid or dihomo-γ-linolenic acid can be more effectively accumulated by culturing with the addition of said omega 6 unsaturated fatty acid that is precursor of arachidonic acid or dihomo-γ-linolenic acid among the fatty acids. As the omega 6 unsaturated fatty acids, there can be mentioned linoleic acid, γ-linolenic acid, dihomo-γ-linolenic acid, and the like, and as lipids containing said fatty acids as components there can be mentioned safflower oil, soybean oil, corn oil, cottonseed oil, Bio-γ(γ-linolenic acid-containing triglyceride), and the like.

The total amount of the substrate added is 0.001 to 10% by weight and preferably 0.5 to 10% by weight relative to the medium. These substrates may be added either before or immediately after inoculating the producer microorganism, or after the start of culturing, or they may be added at both time points. The addition after the start of culturing may be once, or more than once on an intermittent basis. Alternatively, they may be added continuously. These substrates may be added as the sole carbon source for culturing.

By culturing as described above, lipids containing

arachidonic acid or dihomo-γ-linolenic acid can be formed and accumulated in large quantities in the cells. When liquid medium is used, arachidonic acid or dihomo-γ-linolenic acid can be harvested from the cultured cells as described below.

After culturing, cultured cells may be obtained from the culture by conventionally used means of separating a solid and a liquid such as centrifugation, filtration and the like. The cells are extensively washed with water, and preferably dried. Drying can be effected by lyophilization, air drying, and the like. Dried cells are preferably extracted with an organic solvent under a stream of nitrogen. As organic solvents, an ether, hexane, methanol, ethanol, chloroform, dichloromethane, petroleum ether, and the like can be used, and satisfactory results can also be obtained by alternate extraction with methanol and petroleum ether, or by extraction with a single layer solvent of chloroform-methanol-water. Evaporation of organic solvent from the extract under reduced pressure yields lipids containing arachidonic acid or dihomo-γ-linolenic acid.

Instead of the above-mentioned methods, wet cells may be used for extraction. In cases like this, solvents miscible with water such as methanol and ethanol, or mixed solvents miscible with water comprising these and water and/or other solvents may be used. The other procedures are similar to those described above.

In the lipids obtained as described above, various fatty acids are contained as components of lipid compounds, for example fat. They can be directly separated, but preferably they are separated as esters with a lower alcohol, such as γ-linolenic acid methyl, dihomo-γ-linolenic acid methyl, arachidonic acid methyl, and the like.

By converting into these esters, they can be readily

separated from other lipid components, and can be readily separated from other fatty acids such as palmitic acid and oleic acid (these are also esterified at the same time as arachidonic acid or dihomo-γ-linolenic acid is esterified) that are formed in the culture. For example, in order to obtain a methyl ester of arachidonic acid or dihomo-γ-linolenic acid, the above extracted lipids are preferably treated with dry methanol-hydrochloric acid 5-10%, BF_3-methanol 10-15%, and the like at room temperature for 1-24 hours.

In order to recover arachidonic acid or dihomo-γ-linolenic acid from the above treated solution, extraction is preferably effected with such organic solvents as hexane, ether, ethyl acetate, and the like. The extract is then dried with anhydrous sodium sulfate and the organic solvents are preferably evaporated under reduced pressure to obtain a mixture predominantly composed of fatty acid esters. This mixture contains, in addition to the desired arachidonic acid or dihomo-γ-linolenic acid, fatty acid methyl esters such as palmitic acid methyl ester, stearic acid methyl ester, and oleic acid methyl ester. In order to isolate methyl ester of arachidonic acid or dihomo-γ-linolenic acid from mixtures of these fatty acid methyl esters, column chromatography, low temperature crystallization, urea inclusion, liquid-liquid countercurrent extraction can be used either singly or in combination.

In order to obtain arachidonic acid or dihomo-γ-linolenic acid from these methyl esters of arachidonic acid or dihomo-γ-linolenic acid, they are hydrolyzed in an alkali followed by extraction with an organic solvent such as ether and ethyl acetate.

Furthermore, in order to obtain arachidonic acid or dihomo-γ-linolenic acid without via methyl esters thereof, the above extract is decomposed with an alkali

(e.g. 2 to 3 hours with 5% aqueous solution of sodium hydroxide), and then the decomposed material is subjected to extraction and purification according to the methods conventionally used for the extraction and purification of fatty acids.

The present invention will now be explained in more details with reference to specific examples.

Examples

Example 1.

Mortierella alpina IFO8568 was inoculated into a large slant bottle containing 300 ml of Czapek agar medium (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, 3% sucrose, 2% agar, pH 6.0), and was cultured at 28°C for 2 weeks. After culturing, 50 ml of sterile water to which had been added 2 drops of Tween80 was added to the large slant bottle, which was shaken sufficiently, and then filtered with 4 ply gauze. This procedure was repeated twice, and the filtrate was centrifuged at 8000 x g for 10 minutes. Spores thus obtained were suspended into Tris/maleate buffer solution (pH 7.5) to 1 x 10^6 /ml to prepare a spore solution.

To 1.0 ml of the spore solution thus obtained, 0.5 ml of 100 mM Tris/maleate buffer solution (pH 7.5) was added, and 500 μg of the NTG solution (N-methyl-N'-nitro-N-nitrosoguanidine 5 mg/deionized water 1 ml) was added, which was subjected to mutagenesis by incubating at 28°C for 15 minutes. After adding 3 ml of 10% $Na_2S_2O_3$, the reaction mixture was centrifuged at 5500 x g for 10 minutes, and the precipitate (spores subjected to mutagenesis) was washed with 3 ml of sterile water and centrifuged at 5500 x g for 10 minutes, to which 2 ml of sterile water was added to prepare a NTG-treated spore suspension.

The NTG-treated spore suspension was diluted to about 10^{-3} to 10^{-4} and then plated on a GY agar plate (1%

glucose, 0.5% yeast extract, 0.05% Triton X-100, 1.5% agar, pH 6.0). After incubating at 28°C, developed colonies were randomly picked up, and were cultured at 28°C until growth can be seen, and after the growth was observed, the culture was stored.

The stored colonies that were picked up were cultured on a GY agar plate at 28°C for 2 days and at 12°C for 2 days, and then were punched out together with the agar prior to drying at 100°C. The dried cells obtained were placed into a screw capped test tube (16.5 mm ϕ), to which 1 ml of methylene chloride and 2 ml of dry methanol-hydrochloric acid (10%) were added and were treated at 50°C for 3 hours to methylesterify them. Four ml of n-hexane and 1 ml of water were added thereto, and extracted twice. After the solvent of the extract was evaporated using a centrifuge evaporator (40°C, 1 hour), the fatty acid methyl ester obtained was analyzed by capillary gas chromatography. After screening, Mortierella alpina SAM2153 (FERM P-15767) (FERM BP-6794) that does not produce eicosapentaenoic acid at a low temperature culture was obtained.

Example 2.

To a GY agar plate (1% glucose, 0.5% yeast extract, 0.05% Triton X-100, 1.5% agar, pH 6.0), Mortierella alpina IFO8568 and Mortierella alpina SAM2153 (FERM P-15767) (FERM BP-6794) obtained in Example 1 were separately inoculated and were subjected to stationary culture. The culture temperature comprised the following 6 conditions:

1. 28°C (2 days), 12°C (2 days)
2. 28°C (4 days)
3. 12°C (6 days)
4. 28°C (4 days), 12°C (3 days)
5. 28°C (7 days)
6. 12°C (7 days)

After culturing, methyl esterification was conducted as in Example 1, and the fatty acid methyl ester thus

obtained was analyzed by capillary gas chromatography. The results are shown in Table 1.

Table 1. Comparison of fatty acid composition of Mortierella alpina IFO8568 and SAM2153

Culture condition (Days)		Strain	Fatty acid composition (%)									
28°C	12°C		16:0	18:0	18:1ω9	18:2ω6	18:3ω6	DGLA	Ara	EPA	24:0	Others
2	2	IFO 8568	16.67	11.79	19.34	5.98	3.71	4.60	25.61	1.11	2.90	8.29
2	2	SAM 2153	15.30	12.89	16.17	6.52	4.91	5.61	28.97	0	2.85	6.78
4	0	IFO 8568	16.04	12.28	15.78	6.76	3.89	3.56	29.66	0	3.38	8.65
4	0	SAM 2153	18.63	12.27	17.19	8.37	4.36	4.49	24.59	0	3.25	6.85
0	6	IFO 8568	12.77	11.27	13.14	5.43	4.50	6.43	33.89	3.02	2.09	7.46
0	6	SAM 2153	12.65	12.31	11.61	5.90	4.84	7.08	37.02	0	1.89	6.70
4	3	IFO 8568	12.36	8.06	14.63	6.78	4.76	4.20	39.58	0	3.27	6.36
4	3	SAM 2153	11.57	7.98	11.12	6.55	4.76	5.47	43.01	0	2.99	6.55
7	0	IFO 8568	10.85	7.43	11.99	7.02	4.66	4.18	42.46	0	4.64	6.77
7	0	SAM 2153	12.96	8.64	13.06	8.47	4.47	4.12	39.36	0	3.30	5.62
0	7	IFO 8568	12.48	8.16	14.94	6.43	5.10	7.82	37.18	3.31	0.57	4.01
0	7	SAM 2153	10.42	9.66	8.63	5.62	4.64	7.08	47.28	0	1.65	5.02

16:0, palmitic acid; 18:0, stearic acid; 18:1ω9, oleic acid; 18:2ω6, linoleic acid; 18:3ω6, γ-linolenic acid; DGLA, dihomo-γ-linolenic acid; Ara, arachidonic acid; EPA, eicosapentaenoic acid; 24:0, tetracosanoic acid

The parent strain IFO8568, when cultured at 12°C, produced eicosapentaenoic acid, and its ratio increased in proportion with the culturing time at 12°C, whereas the mutant strain Mortierella alpina SAM2153 (FERM P-15767) (FERM BP-6794) did not produce eicosapentaenoic acid at all even by culturing for a long time at 12°C, revealing that it is a mutant strain in which the activity Of ω3 desaturase (enzymes that convert arachidonic acid to eicosapentaenoic acid) is lacking or extremely decreased. Also, since it was not capable of converting arachidonic acid to eicosapentaenoic acid, it was found that the content of arachidonic acid could be efficiently enhanced by culturing at a low temperature because arachidonic acid that otherwise would have been converted to eicosapentaenoic acid was accumulated by culturing at a low temperature.

Example 3.

Two ml of a medium (pH 6.0) containing 4% glucose and 1% yeast extract was placed in a 10 ml Erlenmeyer flask and was sterilized at 120°C for 20 minutes. One platinum loopful each of Mortierella alpina IFO8568 and Mortierella alpina SAM2153 (FERM P-15767) (FERM BP-6794) obtained in Example 1 were inoculated on the medium, and were cultured under shaking using a reciprocating shaker (150 rpm) at 12°C for 7 days, or at 12°C for 10 days. The results are shown in Table 2. It was confirmed in the liquid culture also that no eicosapentaenoic acid was produced, even by culturing at 12°C, resulting in an enhanced ratio and an increased amount of arachidonic acid produced.

Table 2 Comparison of fatty acid composition of and the amount of arachidonic acid produced by Mortierella alpina IFO8568 and SAM2153

Culture condition Temp.	Days	Strain	Degree of growth (g/l)	Amount of Ara produced (g/l)	Fatty acid composition (%)									
					16:0	18:0	18:1ω9	18:2ω6	18:3ω6	DGLA	Ara	EPA	24:0	Others
12°C	7	IFO 8568	9.48	0.28	12.92	8.14	19.17	7.79	6.75	6.84	28.21	3.41	1.02	5.75
		SAM 2153	10.52	0.48	11.47	11.65	12.30	6.14	6.93	9.58	36.12	0	1.65	4.16
12°C	10	IFO 8568	11.50	0.72	13.42	7.98	19.80	5.78	5.57	7.17	30.73	3.50	1.12	4.93
		SAM 2153	12.88	1.41	8.71	11.85	7.75	3.95	5.79	8.51	48.22	0	2.03	3.19

16:0, palmitic acid; 18:0, stearic acid; 18:1ω9, oleic acid; 18:2ω6, linoleic acid; 18:3ω6, γ-linolenic acid; DGLA, dihomo-γ-linolenic acid; Ara, arachidonic acid; EPA, eicosapentaenoic acid; 24:0, tetracosanoic acid

Example 4.

To 2 ml of a medium (pH 6.0) containing 4% glucose and 1% yeast extract, substrates for arachidonic acid biosynthesis or lipids containing them shown in Table 3 were each added at 0.5%, and the mixture was placed in a 10 ml Erlenmeyer flask and was sterilized at 120°C for 20 minutes. One platinum loopful of Mortierella alpina SAM2153 (FERM P-15767) (FERM BP-6794) obtained in Example 1 was inoculated on the medium, and was cultured under shaking using a reciprocating shaker (150 rpm) at 12°C for 10 days. The results are shown in Table 3.

Table 3

Additive	Arachidonic acid		
	Content (%)	Amount produced (g/l)	(mg/g)
No addition	48.22	1.41	109.2
Octadecane	49.23	1.46	111.2
Sodium oleate	50.10	1.56	119.3
Sodium linoleate	51.30	1.63	124.3
Sodium linolenate	52.71	1.65	123.5
Methyl oleate	52.92	1.68	127.1
Methyl linoleate	53.20	1.73	128.4
Methyl linolenate	53.25	1.74	128.6
Soybean oil	54.06	1.81	129.0
Corn oil	53.76	1.81	131.4
Cottonseed oil	54.88	1.84	130.9
Safflower oil	56.64	2.05	143.7

Example 5.

Five liters of a medium (pH 6.0) containing 2% glucose, 1.5% soy flour protein, 0.3% KH_2PO_4, 0.05% $MgCl_2 \cdot 6H_2O$, 0.1% Na_2SO_4, and 0.1% soybean oil was placed in a 10 L jar fermentor and was sterilized at 120°C for 30 minutes. Mortierella alpina SAM2153 (FERM P-15767) (FERM BP-6794) obtained in Example 1 was inoculated and was subjected to an airation and agitation culture at an aeration rate of 1 vvm for 10 days. The culture temperature was 20°C at the start of culturing, and from day 3 it was gradually decreased to 12°C. Only on day 1, was 1% glucose added.

From day 2, 12 ml of the culture was sampled out, was methylesterified, and the fatty acid methyl esters obtained were analyzed by gas chromatography. Figures 1a, b, c, and d each show changes in the amount (g/l) of arachidonic acid produced, the ratio (%) of arachidonic acid to the total fatty acids, the degree of growth (g/l), and glucose concentrations (%) in the medium. Culturing at a low temperature in a 10 L jar fermentor was confirmed and surprisingly the ratio of arachidonic acid to the total fatty acids reached as high as 56.4% on day 10 of culturing. Analysis of lipid fractions in the intracellular lipids on day 10 of culturing revealed that triacylglycerol was 85.8%, free fatty acids 2.1%, diacylglycerol 0.5%, phosphatidyl ethanolamine 3.8%, phosphatidyl choline 3.9%, phosphatidyl serine 2.0%, and phosphatidic acid 1.9%.

Example 6.

Five liters of a medium (pH 6.0) containing 2% glucose, 1.5% soy flour, 0.3% KH_2PO_4, 0.05% $MgCl_2 \cdot 6H_2O$, 0.05% $CaCl_2 \cdot 2H_2O$, 0.1% Na_2SO_4, and 0.1% soybean oil was placed in a 10 L jar fermentor and was sterilized at 120°C for 30 minutes.

On the other hand, Mortierella alpina IFO8568 as a parent strain in a similar manner to Example 1, was subjected to mutation treatment again to obtain Mortierella alpina SAM2239, a strain in which ω3 desaturase activity was decreased.

This SAM2239 was inoculated and was subjected to an airation and agitation culture at an aeration rate of 1 vvm for 12 days. The culture temperature was 24°C at the start of culturing, and from day 3 it was gradually decreased to 12°C. On day 1, 2, and 3, 1% glucose was added. On day 12, the final day of culturing, sampling was carried out, and the fatty acid methyl esters obtained were analyzed by gas chromatography. The results indicated that the ratio of arachidonic acid to

the total fatty acids reached as high as 75.1% and the amount produced thereof was 4.1 g/l.

Reference to the microorganisms deposited under the Patent Cooperation Treaty, Rule 13-2, and the name of the Depository Authority

Depository Authority:

Name: the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology

Address: 1-3, Higashi 1-chome, Tsukuba city, Ibaraki pref., Japan

Microorganism (1)

Name: Mortierella elongata SAM0219

Accession number: FERM BP-1239

Deposition Date: March 19, 1986

Microorganism (2)

Name: Mortierella alpina SAM2153

Accession number: FERM BP-6794

Deposition Date: August 5, 1996

CLAIMS

1. A process for producing lipids containing arachidonic acid comprising the steps of

culturing a microorganism in which ω3 desaturase activity has been decreased or is lacking at a temperature lower than the optimum growth temperature from the start of culturing or after culturing at the optimum growth temperature, said microorganism being obtained by mutagenesis of a microorganism capable of producing arachidonic acid and belonging to the genus Mortierella, the genus Conidiobolus, the genus Pythium, the genus Phytophthora, the genus Penicillium, the genus Cladosporium, the genus Mucor, the genus Fusarium, the genus Aspergillus, the genus Rhodotorula, the genus Entomophthora, the genus Echinosporangium or the genus Saprolegnia; and then

recovering lipids containing arachidonic acid from the culture.

2. The process for producing lipids containing arachidonic acid according to claim 1 comprising culturing said mutant strain in a medium containing hydrocarbons, fatty acids, fatty acid esters, fatty acid salts, or lipids containing them as components; or adding to the culture in which said mutant strain is being cultured hydrocarbons, fatty acids, fatty acid esters, fatty acid salts, or lipids containing them as components, and then further culturing.

3. A process for producing lipids containing arachidonic acid comprising the steps of

culturing a microorganism in which ω3 desaturase activity has been decreased or is lacking at a temperature lower than 20°C from the start of culturing or after culturing at 20 to 40°C, said microorganism being obtained by the mutagenesis of a microorganism belonging to the subgenus Mortierella; and then

recovering lipids containing arachidonic

acid from the culture.

4. The method of producing lipids containing arachidonic acid according to claim 3 comprising culturing said mutant strain in a medium containing hydrocarbons, fatty acids, fatty acid esters, fatty acid salts, or lipids containing them as components; or adding to the culture in which said mutant strain is being cultured hydrocarbons, fatty acids, fatty acid esters, fatty acid salts, or lipids containing them as components, and then further culturing.

5. A method of producing lipids containing dihomo-γ-linolenic acid comprising the steps of

culturing a microorganism in which ω3 desaturase activity has been decreased or is lacking at a temperature lower than the optimum growth temperature from the start of culturing or after culturing at the optimum growth temperature, said microorganism being obtained by the mutagenesis of a microorganism capable of producing arachidonic acid and belonging to the genus Mortierella, the genus Conidiobolus, the genus Pythium, the genus Phytophthora, the genus Penicillium, the genus Cladosporium, the genus Mucor, the genus Fusarium, the genus Aspergillus, the genus Rhodotorula, the genus Entomophthora, the genus Echinosporangium or the genus Saprolegnia; and then

recovering lipids containing dihomo-γ-linolenic acid from the culture.

6. The method of producing lipids containing dihomo-γ-linolenic acid according to claim 5 wherein said mutant strain is a mutant strain in which further Δ5 desaturase activity has been decreased or is lacking.

7. The method of producing lipids containing dihomo-γ-linolenic acid according to claim 5 or 6 comprising culturing said mutant strain in a medium containing hydrocarbons, fatty acids, fatty acid esters,

fatty acid salts, or lipids containing them as components; or adding to the culture in which said mutant strain is being cultured hydrocarbons, fatty acids, fatty acid esters, fatty acid salts, or lipids containing them as components, and then further culturing.

8. A method of producing lipids containing dihomo-γ-linolenic acid comprising the steps of

culturing a microorganism in which ω3 desaturase activity has been decreased or is lacking at a temperature lower than 20°C from the start of culturing or after culturing at 20 to 40°C, said microorganism being obtained by the mutagenesis of a microorganism belonging to the subgenus Mortierella; and then

recovering lipids containing dihomo-γ-linolenic acid from the culture.

9. The method of producing lipids containing dihomo-γ-linolenic acid according to claim 8 wherein said mutant strain is a mutant strain in which further Δ5 desaturase activity has been decreased or is lacking.

10. The method of producing lipids containing dihomo-γ-linolenic acid according to claim 8 or 9 comprising culturing said mutant strain in a medium containing hydrocarbons, fatty acids, fatty acid esters, fatty acid salts, or lipids containing them as components; or adding to the culture in which said mutant strain is being cultured hydrocarbons, fatty acids, fatty acid esters, fatty acid salts, or lipids containing them as components, and then further culturing.

11. An arachidonic acid-containing microbial lipid containing 72% by weight or more of arachidonic acid to the total fatty acids in said lipid.

12. The arachidonic acid-containing microbial lipid according to claim 11 wherein the percentage of eicosapentaenoic acid to the total fatty acids in said lipid is 0.5% by weight or less.

13. A microorganism wherein ω3 desaturase activity has been decreased or is lacking, said microorganism being obtained by the mutagenesis of a microorganism capable of producing arachidonic acid and belonging to the genus Mortierella, the genus Conidiobolus, the genus Pythium, the genus Phytophthora, the genus Penicillium, the genus Cladosporium, the genus Mucor, the genus Fusarium, the genus Aspergillus, the genus Rhodotorula, the genus Entomophthora, the genus Echinosporangium or the genus Saprolegnia.

14. The microorganism according to claim 13 wherein ω3 desaturase activity has been decreased or is lacking, wherein said microorganism capable of producing arachidonic acid that is subjected to mutagenesis is a microorganism belonging to the genus Mortierella subgenus Mortierella.

15. The microorganism according to claim 14 wherein ω3 desaturase activity has been decreased or is lacking, wherein said microorganism, capable of producing arachidonic acid, that is subjected to mutagenesis is Mortierella alpina.

16. The microorganism according to claim 15 wherein ω3 desaturase activity has been decreased or is lacking, wherein said microorganism wherein ω3 desaturase activity has been decreased or lacked is Mortierella alpina SAM2153 (FERM BP-6794).

17. A lipid containing arachidonic acid wherein the arachidonic acid content in the total fatty acids in the lipid is 50% by weight or more, said lipid being obtained by culturing a microorganism according to any one of claims 13 to 16 in which ω3 desaturase activity has been decreased or is lacking.

18. A lipid containing arachidonic acid in which the arachidonic acid content in the total fatty acids in the lipid is 50% by weight or more and the

eicosapentaenoic acid content is 0.5% by weight or less, said lipid being obtained by culturing a microorganism according to any one of claims 13 to 16 in which ω3 desaturase activity has been decreased or is lacking.

19. A lipid containing arachidonic acid according to claim 17 or 18 wherein the arachidonic acid content in the total fatty acids in the lipid is 60% by weight or more.

20. A lipid containing arachidonic acid according to claim 17 or 18 wherein the arachidonic acid content in the total fatty acids in the lipid is 70% by weight or more.

ABSTRACT

A process for producing lipids containing arachidonic acid comprising culturing a microorganism in which ω3 desaturase activity has been decreased or is lacking at a temperature lower than the optimum growth temperature from the start of culturing or after culturing at the optimum growth temperature, said microorganism being obtained by the mutation treatment of a microorganism capable of producing arachidonic acid and belonging to the genus Mortierella and the like; and then recovering lipids containing arachidonic acid from the culture.

Declaration and Power of Attorney For Patent Application

特許出願宣言書及び委任状

Japanese Language Declaration

日本語宣言書

下記の氏名の発明者として、私は以下の通り宣言します。

As a below named inventor, I hereby declare that:

私の住所、私書箱、国籍は下記の私の氏名の後に記載された通りです。

My residence, post office address and citizenship are as stated next to my name.

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I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PROCESS FOR PRODUCING ARACHIDONIC ACID-CONTAINING LIPIDS AND DIHOMO-γ-LINOLENIC ACID-CONTAINING LIPIDS

上記発明の明細書（下記の欄でx印がついていない場合は、本書に添付）は、

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☐ was filed on August 27, 1999
as United States Application Number or
PCT International Application Number
PCT/JP99/04653 and was amended on
_____ (if applicable).

私は、特許請求範囲を含む上記訂正後の明細書を検討し、内容を理解していることをここに表明します。

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

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Japanese Language Declaration
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私は、米国法典第３５編１１９条(a)-(d)項又は３６５条(b)項に基き下記の、米国以外の国の少なくとも一ヵ国を指定している特許協力条約３６５(a)項に基ずく国際出願、又は外国での特許出願もしくは発明者証の出願についての外国優先権をここに主張するとともに、優先権を主張している、本出願の前に出願された特許または発明者証の外国出願を以下に、枠内をマークすることで、示しています。

I hereby claim foreign priority under Title 35, United States Code, Section 119 (a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s) 外国での先行出願 (Number) (番号)	(Country) (国名)	(Day/Month/Year Filed) (出願年月日)	Priority Not Claimed 優先権主張なし
10-243583(Pat. Appln.)	Japan	28/August/1998	☐
			☐

私は、第３５編米国法典１１９条(e)項に基いて下記の米国特許出願規定に記載された権利をここに主張いたします。

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

(Application No.) (出願番号)	(Filing Date) (出願日)	(Application No.) (出願番号)	(Filing Date) (出願日)

私は、下記の米国法典第３５編１２０条に基いて下記の米国特許出願に記載された権利、又は米国を指定している特許協力条約３６５条(c)に基ずく権利をここに主張します。また、本出願の各請求範囲の内容が米国法典第３５編１１２条第１項又は特許協力条約で規定された方法で先行する米国特許出願に開示されていない限り、その先行米国出願書提出日以降で本出願書の日本国内または特許協力条約国際提出日までの期間中に入手された、連邦規則法典第３７編１条５６項で定義された特許資格の有無に関する重要な情報について開示義務があることを認識しています。

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s), or 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of application.

(Application No.) (出願番号)	(Filing Date) (出願日)	(Status: Patented, Pending, Abandoned) (現況：特許許可済、係属中、放棄済)

私は、私自身の知識に基ずいて本宣言書中で私が行なう表明が真実であり、かつ私の入手した情報と私の信じるところに基ずく表明が全て真実であると信じていること、さらに故意になされた虚偽の表明及びそれと同等の行為は米国法典第１８編第１００１条に基ずき、罰金または拘禁、もしくはその両方により処罰されること、そしてそのような故意による虚偽の声明を行なえば、出願した、又は既に許可された特許の有効性が失われることを認識し、よってここに上記のごとく宣誓を致します。

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Japanese Language Declaration
(日本語宣言書)

委任状： 私は下記の発明者として、本出願に関する一切の手続きを米特許商標局に対して遂行する弁理士または代理人として、下記の者を指名いたします。（弁護士、または代理人の氏名及び登録番号を明記のこと）

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith *(list name and registration number)*

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